Eur. J. Clin. Chem. Clin. Biochem.
Vol. 30, 1992, pp. 223–228

Determination of Apolipoprotein B in Apolipoprotein CII/CIII-Containing Lipoproteins by an Immunoenzymmetric Assay[1])

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(Received September 17, 1991/February 6, 1992)

Summary: A solid phase sandwich immunoenzymmetric assay is described for the determination of apolipoprotein B in apolipoprotein CII- and/or CIII-containing lipoproteins (chylomicron remnants, VLDL, IDL). During a first incubation step the particles containing apolipoproteins CII and/or CIII are bound to antibodies against these components, while the antibodies are immobilised on microtitre plate wells. After washing, anti-apolipoprotein B is added in a second incubation step. Finally peroxidase-labelled anti-sheep IgG reacts with the bound anti-apolipoprotein B, thus allowing the quantification of complexes containing both B and CII/CIII apolipoproteins.

The amounts of these complexes were correlated with total cholesterol, triacylglycerols, HDL- and LDL-cholesterol, apolipoproteins AI and B, as well as with age and sex of the subject. A total of 258 individuals was studied, including patients with lipid metabolism disorders, patients with manifest coronary heart disease, and healthy controls.

The assay described in this article was compared with radial immunodiffusion after pretreatment of samples. The immunoenzymmetric assay was easier and faster to perform and had a lower detection limit than the classical VLDL apolipoprotein B determination using ultracentrifugation. Furthermore, it could be performed directly on native serum.

Most importantly, the study revealed elevated apolipoprotein CII/CIII-B levels in coronary heart disease patients of both sexes compared with normal subjects. Furthermore, in male coronary heart disease patients a negative correlation was found between the concentrations of apolipoprotein CII/CIII-B and HDL-cholesterol. These results suggest a delayed VLDL-HDL exchange of lipids and proteins in these patients which results in an accumulation of atherogenic apolipoprotein B-containing VLDL and IDL.

Introduction

The total serum concentration of apolipoprotein B is a well known risk factor for premature coronary heart disease. LDL enriched in apolipoprotein B relative to lipids are known to be especially atherogenic (1–11). However, in hypertriglyceridaemic sera, up to 50% of total apolipoprotein B are present in VLDL and chylomicrons as well as in their remnants (12–16). Although an increase in VLDL concentration does not appear to represent a primary risk factor for coronary heart disease, some studies have revealed an association between increased concentrations of this lipoprotein and premature development of atherosclerosis (17–19). The special role played by VLDL in the development of atherosclerosis may lie in the altered composition of these particles in patients when compared with healthy controls (20, 21). Hence it is

[1]) Funding organisation: Ministerium für Wissenschaft, Forschung und Technik Nordrhein-Westfalen, IV B 5-40001986

of interest to quantify the concentration of apolipoprotein B in VLDL. To date, the measurement of this analyte requires time-consuming analysis with ultracentrifugation or lengthy sample preparation (22, 23). Due to these obstacles, epidemiological or larger clinical studies have not taken this important quantity into consideration.

We present here an immunoenzymmetric quantitation of apolipoprotein CII/CIII-apolipoprotein B which can be performed within one working day, without ultracentrifugation and pretreatment of samples. Furthermore, native lipoprotein particles are used, which eliminates disturbances due to artificial changes. This technique is based on the fact that VLDL are the only lipoproteins in human serum which contain C apolipoproteins as well as apolipoprotein B, with the exception of relatively small amounts of chylomicron remnants and IDL. In a first step, all apolipoprotein CII/CIII-containig lipoproteins are linked to the fixed phase. In the second step, the apolipoprotein B contained in these particles is quantified.

Materials and Methods

Samples

In this study we investigated a total of 258 subjects who belonged to the following groups:

(a) normal individuals: 90 male and 31 female subjects of the PROCAM study pool (24) showing normal lipid and lipoprotein patterns, and fasting glucose concentrations within the normal range (3.33 – 6.05 mmol/l)

(b) patients with coronary heart disease: 61 male and 18 female patients of the LVA Fachklinik Salzetal (Head: Prof. Dr. *E. Köhler*) with coronary atherosclerosis as confirmed by coronary angiography

(c) subjects with lipid metabolism disorders: 35 male and 23 female patients from the outpatient clinic of the Institute of Clinical Chemistry and Laboratory Medicine, University of Münster, presenting with various forms of dyslipidaemia.

Measurements were done on large series of frozen serum samples, which were frozen at −70 °C for a maximum of 6 months. Stability of the samples for at least 6 months had been ascertained previously in a comparative study of 30 samples.

Methods

Raising of antibodies and preparation for assay

Apolipoproteins CII and CIII were isolated by preparative isoelectric focusing of VLDL apolipoproteins, which were obtained by ultracentrifugation and subsequent delipidation (25). Anti-apolipoprotein CII and anti-apolipoprotein CIII antisera were raised in two New Zealand white rabbits by subcutaneous injection of 1 mg of the respective antigen in complete *Freund*'s adjuvant, followed after six weeks with a booster injection of a 0.5 mg apolipoprotein in incomplete *Freund*'s adjuvant. Starting at the third week after boosting, 40 ml blood was taken from the auricular artery once a week from each rabbit. The antisera were tested for monospecifity by *Ouchterlony* diffusion, two-dimensional electroimmunodiffusion, and Western blotting against isolated lipoproteins as well as purified apolipoproteins. In the immunoenzymmetric assay a mixture of equal amounts of chromatographically (DEAE-sepharose) purified IgG fractions (26) of anti-apolipoprotein CII and anti-apolipoprotein CIII antibodies was used for coating. For the detection step, sheep anti-apolipoprotein B antibodies (Boehringer Mannheim) and horseradish peroxidase-labelled anti-sheep IgG (Dako) were used. The anti-apolipoprotein C antibodies were stored at −20 °C, anti-apolipoprotein B antibodies and the enzyme conjugate at + 4 °C.

Lipid analysis

Total cholesterol and triacylglycerols were determined enzymatically with the SMAC autoanalyser (Technicon GmbH) as previously described (27). HDL-cholesterol was measured following precipitation with phosphotungstic acid/$MgCl_2$ (28) using the COBAS Bio centrifugal analyser (Roche). LDL-cholesterol was calculated using the *Friedewald* formula (29).

Apolipoprotein analysis

Apolipoproteins AI and B were measured turbidimetrically, as previously described (30). For the determination of apolipoprotein CII/CIII-apolipoprotein B, microtitre plates from Dynatech were selected, because specific binding of antibodies during coating was high and unspecific binding during the following incubation steps was low, compared with other microtitre plates (Costar, Flow, NUNC). No interlot variations were observed using three different lots of Dynatech plates. The microtitre plates were coated with the optimal concentration of 0.1 µg anti-apolipoprotein CII + 0.1 µg anti-apolipoprotein CIII per well, which had been determined earlier in a dilution series using a checker board system. Coating was performed overnight in carbonate buffer (0.1 mol/l, pH 9.6), followed by washing 5 times with 0.15 mmol/l NaCl containing 50 µl/l Tween 20. A sample (100 µl) containing serum and phosphate buffered saline (0.1 mol/l, pH 7.4) in a ratio 1 : 100 or 1 : 200 was placed in each well of the microtitre plate and incubated for two hours. After washing 5 times with phosphate buffered saline, 1 µg anti-apolipoprotein B antibody (Boehringer Mannheim), diluted 1 : 20000 in phosphate buffered saline was added to each well and incubated for two hours. Subsequently the plates were again washed 5 times with phosphate buffered saline and then incubated for two hours with peroxidase-labelled anti-sheep antibody diluted 1 : 1000 in phosphate buffered saline. All incubation steps were carried out at room temperature. After another washing step, 100 µl *o*-phenylenediamine/H_2O_2 were added as substrate. The colour reaction was stopped after half an hour by addition of 100 µl 1 mol/l H_2SO_4. A Dynatech MR 600 microtitre reader coupled to a computer was used for data reduction.

Intra- and inter-assay variation

The intra-assay variations amounted to 7.5% and 5.5% (n = 20) and the inter-assay variations were 10.4% and 6.7% (n = 30) at concentrations of 0.08 g/l and 0.16 g/l apolipoprotein CII/CIII-B, respectively. The linear range extended from 0.01 g/l to 0.5 g/l. The sensitivity of the assay was 10 µg/l apolipoprotein CII/CIII-B. Disturbances by extremely high concentrations of triacylglycerols (> 6.8 mmol/l) or cholesterol (> 10.4 mmol/l) were not observed.

Calibration

The assays were calibrated with a dilution series of a normolipaemic pool serum. After determination of total apolipoprotein B using turbidimetry, all CII/CIII-containing lipoproteins were removed by antibody-mediated fixed phase immune ad-

Tab. 1. Distribution of age and lipid quantities in normal subjects

	Males (n = 90)			Females (n = 31)		
	mean	median	S. D.	mean	median	S. D.
Age (years)	42.4	41.8	10.6	38.6	36.3	11.0
Cholesterol (mmol/l)	5.5	5.3	1.1	5.5	5.4	1.1
Triacylglycerols (mmol/l)	1.6*	1.2	1.1	1.0	1.0	0.4
HDL-cholesterol (mmol/l)	1.1*	1.1	0.2	1.6	1.6	0.4
LDL-cholesterol (mmol/l)	3.6	3.6	1.0	3.4	3.1	1.1
Apolipoprotein AI (g/l)	1.36*	1.36	0.19	1.69	1.67	0.28
Apolipoprotein B (g/l)	0.86	0.85	0.20	0.78	0.80	0.20
Apolipoprotein CII/CIII-B (g/l)	0.16	0.14	0.07	0.16	0.14	0.07

* significantly different when compared with female subjects, $p < 0.05$

sorption on nylon membranes (Nalgene). Subsequently apolipoprotein B was remeasured in the remainder. The amount of apolipoprotein CII/CIII-B was calculated from the difference between this latter value and the total apolipoprotein B value.

Statistical analysis

For the statistical evaluation of the data the "Statistical Package for the Social Sciences" (SPSS^x) was used (31). The *Mann-Whitney* U-test was applied for a comparison of parameter distribution in different collectives. The parameter independent *Spearman*-test was used for the calculations of correlations whithin the different subgroups, the *Kruskal-Wallis*-Test for comparing more than two groups. For all calculations the level of significance was $p < 0.05$.

Tab. 2. Normal subjects: *Spearman* correlation coefficients between age, apolipoprotein CII/CIII-B, and lipid quantities

	Males	Females
Age	0.21*	0.40*
Cholesterol	0.23*	0.43*
Triacylglycerols	0.20*	0.22
HDL-cholesterol	−0.04	−0.25
LDL-cholesterol	0.22*	0.47*
Apolipoprotein AI	−0.03	−0.31*
Apolipoprotein B	0.24*	0.46*

* $p < 0.05$

Results

Distributions and and correlations

Control group

Table 1 describes mean values, median, and standard deviations of the lipid, lipoprotein, and apolipoprotein values measured in the normal collective.

Table 2 describes univariate correlations between apolipoprotein CII/CIII-B concentration and age, concentrations of total cholesterol, triacylglycerols, HDL-cholesterol, LDL-cholesterol, apolipoprotein AI, and apolipoprotein B. In both men and women apolipoprotein CII/CIII-B positively correlated with age, concentrations of total cholesterol, LDL-cholesterol, and apolipoprotein B. Additionally a positive correlation was found between apolipoprotein CII/CIII-B and triacylglycerols in men. Furthermore, a significantly negative correlation was shown between apolipoprotein CII/CIII-B and apolipoprotein AI concentration in women.

Coronary patients

The distribution of lipid quantities measured in the collective of male and female patients with coronary heart disease is shown in table 3. In male patients

Tab. 3. Distribution of age and lipid quantities in coronary heart disease patients

	Males (61)			Females (18)		
	mean	median	S. D.	mean	median	S. D.
Age (years)	54.5*	56.3	9.4	62.1	64.0	7.2
Cholesterol (mmol/l)	6.0	5.8	1.3	6.1	5.7	0.9
Triacylglycerols (mmol/l)	1.7	1.5	0.8	1.8	1.7	0.6
HDL-cholesterol (mmol/l)	1.0*	1.0	0.4	1.3	1.1	0.8
LDL-cholesterol (mmol/l)	4.2	4.0	1.1	4.0	4.0	0.34
Apolipoprotein AI (g/l)	1.24*	1.21	0.23	1.45	1.40	0.18
Apolipoprotein B (g/l)	1.02	0.99	0.20	0.97	0.99	0.09
Apolipoprotein CII/CIII-B (g/l)	0.25	0.22	0.09	0.22	0.21	

* significantly different when compared with female subjects, $p < 0.05$

apolipoprotein CII/CIII-B negatively correlated with HDL-cholesterol and positively with cholesterol, triacylglycerols, LDL-cholesterol, and apolipoprotein B. In female patients, only a correlation between apolipoprotein CII/CIII-B and age was found (tab. 4).

Tab. 4. Coronary heart disease patients: *Spearman* correlation coefficients between age, apolipoprotein CII/CIII-B, and other lipid quantities

	Males	Females
Age	−0.21	−0.42*
Cholesterol	0.37*	0.37
Triacylglycerol	0.30*	−0.14
HDL-cholesterol	−0.29*	0.17
LDL-cholesterol	0.46*	0.37
Apolipoprotein AI	−0.12	0.27
Apolipoprotein B	0.47*	0.12

* $p < 0.05$

Dyslipidaemic patients

In table 5 the statistical description of a group of dyslipidaemic patients is presented. In men we observed a negative correlation between apolipoprotein CII/CIII-B and age, in women a positive correlation between apolipoprotein CII/CIII-B and the concentrations of cholesterol, triacylglycerols, LDL-cholesterol, and apolipoprotein B (tab. 6).

Tab. 6. Hyperlipidaemic subjects: *Spearman* correlation coefficients between age, apolipoprotein CII/CIII-B, and other lipid quantities

	Males	Females
Age	−0.48*	−0.11
Cholesterol	0.17	0.73*
Triacylglycerols	0.03	0.44*
HDL-cholesterol	−0.07	−0.28
LDL-cholesterol	0.09	0.75*
Apolipoprotein AI	−0.11	−0.10
Apolipoprotein B	0.19	0.73*

* $p < 0.05$

Tab. 7. Group-dependent differences in males (group 1: hyperlipidaemic subjects, group 2: normal subjects, group 3: coronary heart disease patients)

	Group 1 (n = 61)	Group 2 (n = 90)	Group 3 (n = 35)
Age (years)	49*,**	42***	55
Cholesterol (mmol/l)	7.6*,**	5.5***	6.0
Triacylglycerols (mmol/l)	2.4*	1.6***	1.7
HDL-cholesterol (mmol/l)	1.1**	1.1***	0.9
LDL-cholesterol (mmol/l)	5.2*,**	3.6***	4.2
Apolipoprotein AI (g/l)	1.35**	1.36***	1.24
Apolipoprotein B (g/l)	1.13*	0.86***	1.02
Apolipoprotein CII/CIII-B (g/l)	0.16**	0.16***	0.26

* significantly different between group 1 and group 2, $p < 0.05$
** significantly different between group 1 and group 3, $p < 0.05$
*** significantly different between group 2 and group 3, $p < 0.05$

Comparison of groups

Table 7 shows a comparison of the results obtained in the 3 different male collectives. Of special interest are the significantly lower HDL-cholesterol and apolipoprotein AI concentrations in the group of coronary heart disease patients compared with the control group and the group of hyperlipidaemic patients. Furthermore, the coronary heart disease patients group presents with significantly higher concentrations of apolipoprotein CII/CIII-B than the other two groups.

Also in the female coronary heart disease patients, HDL-cholesterol and apolipoprotein AI are lower and apolipoprotein CII/CIII-B concentrations are higher, but only in comparison with the normal females (tab. 8).

Tab. 5. Distribution of age and lipid quantities in hyperlipidaemic patients

	Males (35)			Females (23)		
	mean	median	S. D.	mean	median	S. D.
Age (years)	48.8*	51.7	13.7	37.0	32.0	16.5
Cholesterol (mmol/l)	7.6	7.0	2.5	7.2	7.2	2.1
Triacylglycerols (mmol/l)	2.4	1.8	1.9	1.8	1.5	1.1
HDL-cholesterol (mmol/l)	1.1*	1.1	0.4	1.5	1.6	0.5
LDL-cholesterol (mmol/l)	5.2	4.8	1.7	4.8	4.0	2.2
Apolipoprotein AI (g/l)	1.35	1.40	0.37	1.60	1.47	0.41
Apolipoprotein B (g/l)	1.13	1.11	0.44	1.03	1.05	0.39
Apolipoprotein CII/CIII-B (g/l)	0.16	0.14	0.09	0.18	0.17	0.09

* significantly different when compared with female subjects, $p < 0.05$

Tab. 8. Group-dependent differences in females (group 1: hyperlipidaemic subjects, group 2: normal subjects, group 3: coronary heart disease patients)

	Group 1 (n = 18)	Group 2 (n = 31)	Group 3 (n = 23)
Age (years)	37**	39***	62
Cholesterol (mmol/l)	7.2*	5.5***	6.1
Triacylglycerols (mmol/l)	1.8*	1.0***	1.8
HDL-cholesterol (mmol/l)	1.6	1.7***	1.3
LDL-cholesterol (mmol/l)	4.8*	3.4***	4.0
Apolipoprotein AI (g/l)	1.60	1.69***	1.45
Apolipoprotein B (g/l)	1.03	0.78***	0.97
Apolipoprotein CII/CIII-B (g/l)	0.18**	0.15***	0.22

* significantly different between group 1 and group 2, $p < 0.05$
** significantly different between group 1 and group 3, $p < 0.05$
*** significantly different between group 2 and group 2, $p < 0.05$

Discussion

Methods previously available for the quantitation of apolipoprotein B in different lipoproteins were disadvantageous, because their laborious sample preparation, precipitation, or ultracentrifugation precluded their use for the investigation of large numbers of samples. The immunoenzymmetric assay introduced here allows for the quantitation of apolipoprotein B in native particles from serum and permits the economic study of larger collectives. Furthermore, measurements with the immunoenzymmetric assay result in faster and more accurate results than the radial immunodiffusion method, which has been described for the determination of VLDL apolipoprotein B (32). Another important advantage of this new method is that the measured analyte is defined much more accurately by its apolipoprotein composition than lipoproteins obtained by ultracentrifugation or precipitation with polyanions.

Calibration is difficult for both radial immunodiffusion and immunoenzymmetric assays. Radial immunodiffusion is calibrated with an apolipoprotein B value obtained by determination of tetramethyl urea-insoluble proteins in the supernatant after polyvinylsulphate precipitation (32). This procedure requires several steps and therefore easily leads to inaccuracies. The calibration of the immunoenzymmetric assay test only requires the immunoadsorption, and no further preparative or dilution procedures.

It is important to note that the analytes, VLDL apolipoprotein B and apolipoprotein CII/CIII-B, are not identical. VLDL apolipoprotein B concentrations in a control group amount to about 5% of the total apolipoprotein B, whereas apolipoprotein CII/CIII-B amounts to about 20%. This discrepancy is probably caused by co-measurement of chylomicron remnants and IDL in the described immunoenzymmetric test. The atherogenic properties of these lipoproteins are as yet not fully understood.

The possible clinical implication of apolipoprotein CII/CIII-B measurement is highlighted by our observation that apolipoprotein CII/CIII-B concentrations negatively correlate with HDL-cholesterol in male coronary heart disease patients and are significantly higher in coronary heart disease patients than in normal controls. This may reflect a disturbed lipid and protein exchange between VLDL and HDL in coronary heart disease patients, which results in an accumulation of apolipoprotein B-containing VLDL, and hence in a pathogenetic influence on the atherosclerotic process. In male patients with low HDL-cholesterol concentrations, it now appears feasible to use the apolipoprotein CII/CIII-B concentration as an additional value for a more accurate individual risk evaluation.

The results reported here are statistically significant, but their potential clinical importance must be established by larger control studies on an appropriate collective.

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